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Development of multiplex sets of simple sequence repeat DNA markers covering the soybean genome

James M. Narvel[1], Wen-Chy Chu[2], Walter R. Fehr[1,*], Perry B. Cregan[3] & Randy C. Shoemaker[4]

[1]*Dept. of Agronomy, Iowa State University, Ames, IA 50011, USA (*author for correspondence; fax 515-294-6514);* [2]*DNA Sequencing and Synthesis Facility, Iowa State University, Ames, IA, 50011, USA;* [3]*USDA-ARS, Soybean and Alfalfa Research Lab., Beltsville, MD 20705, USA;* [4]*USDA-ARS-CICG, Dept. of Agronomy, Iowa State University, Ames, IA 50011, USA*

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Abstract

Multiplexing involves the analysis of several markers in a single gel lane that is based on the allele size range of marker loci. Multiplex SSR marker analysis is conducted with primers that are labeled with one of three dyes. The development of an SSR multiplex system requires estimates of the allele size range of markers to strategize primer labeling and for grouping markers into multiplex sets. A method is presented that describes the development of multiplex sets of SSR markers in soybean (*Glycine max* (L.) Merr.) by the selective placement of primer sites and by the analysis of diverse germplasm. Primer sites were placed at specific distances from the SSR to adjust the allele size range of marker loci. The analysis of pooled DNA samples comprising diverse soybean genotypes provided robust estimates of the allele size range of marker loci that enabled the development of multiplex sets. Eleven multiplex sets comprising 74 SSR markers distributed across the 20 linkage groups of soybean were developed. Multiplex sets constructed from the analysis of diverse soybean germplasm should have a wide range of genotyping applications. The procedures used in this study were systematic and rapid and should be applicable for multiplex development in any species with SSR marker technology.

Introduction

The value of a DNA marker depends upon its informativeness, genomic abundance, mode of inheritance, and the costs and labor required for analysis [11]. Simple sequence repeat (SSR), or microsatellite markers are composed of a 1 to 6 bp DNA sequence that is repeated a variable number of times [8]. The regions that flank an SSR are usually conserved. This conservation enables the development of complementary primers to these regions for amplifying the intervening SSR by PCR [2]. Variations in the number of times that the SSR is repeated produces PCR products of different length [14]. Genetic analysis of SSRs usually reveals codominant-Mendelian inheritance resulting in a direct relationship between molecular genotype and marker banding pattern (phenotype). This classifies the region amplified by the primers as a locus and PCR-product-length variants as alleles [1, 2]. The utility of SSR markers is attributed to their polymorphic nature, ubiquity throughout eukaryotic genomes, codominant inheritance, and ease of amplification by PCR [10].

Conventional SSR analysis involves amplifying individual markers in separate PCR amplifications, radiolabeling of PCR products, electrophoresis within individual gel lanes, and autoradiographic allele scoring [12]. While this approach is relatively effective, it has limited resolution for discriminating among alleles separated by 1 bp [12]. In addition, the time required for processing gels and scoring autoradiographs and the amount of materials needed for PCR and gel analysis may impose a further limitation.

Technologies have been developed for fluorescent detection of SSR alleles in a semi-automated genotyping system that utilizes dyes for labeling the 5′ end of one of the primer pairs that flank an SSR marker locus [15]. PCR amplification at a marker locus produces multiple copies of an allele, all of which have a dye label on one end. The SSR alleles are analyzed on a DNA sequencer system that detects the dye labels by laser excitation. The dyes emit fluorescence at certain wavelengths, and this information is collected and analyzed by software programs that score SSR alleles as peaks on a graphical display (electropherogram). Internal size standards are run within each lane for accurate allele sizing, even between alleles separated by only 1 bp. Three different dyes can be used as labels to analyze at least three markers in a single gel lane, but this number can be increased if the allele size range of SSR loci is well characterized. Using this information, alleles with the same dye that are run within the same gel lane are correctly scored because they fit the size range of their corresponding SSR locus. Based on these features, multiplexing can be conducted by including several primer pairs in a single PCR amplification (multiplex PCR), by pooling single PCR amplifications, or by combining these approaches. This genotyping system can also be used with amplified fragment length polymorphism (AFLP) markers.

The development of an SSR multiplex system requires information on the allele size range of marker loci for labeling primers and for grouping markers into sets. It is paramount that two markers with overlapping allele size ranges not be labeled with the same dye. Information on allele size ranges may be available from published sources. For example, Reed *et al.* [12] developed 39 chromosome-specific sets comprising 254 SSR markers for human genome analysis by using information from genetic databases. Mitchell *et al.* [9] developed a multiplex set consisting of 11 SSR markers for genotyping in *Brassica* spp. based on previous estimates from conventional analysis. Diwan and Cregan [4] employed 20 fluorescent-labeled SSR markers that covered 11 of the 20 linkage groups of soybean to assay the genetic diversity among 35 ancestral genotypes. They used previous allele size range information to multiplex up to 5 SSRs per gel lane. There have been no reports on the development of multiplex sets of SSR markers without prior knowledge of allele size ranges or of comprehensive sets covering the genome of a crop species. The objective of this study was to develop multiplex sets of SSR markers covering the soybean genome by the selective placement of primer sites and by the analysis of elite and plant introduction (PI) soybean germplasm.

Materials and methods

DNA extraction

A total of 79 soybean genotypes including 40 plant introductions (PIs) and 39 elite cultivars and lines ranging in maturity from Groups I to IV were used in this study. The genotypes were previously used as parents in the formation of soybean populations with a broad genetic base, as described by Fehr and Cianzio [6]. Leaf material (15–20 g) was collected from at least 10 different plants of each genotype. The leaf samples were kept on ice until they were frozen in liquid nitrogen and dried in a vacuum for approximately three days. The dried leaf samples were stored at −20 °C until DNA extraction. The dried leaves were crushed within a plastic bag to obtain a relatively homogeneous sample of all plants within a genotype. About 1 g of crushed leaf material was placed into a 50 ml screw-cap tube containing ca. 4 g of 3 mm glass beads. The leaf material was ground into a powder by agitation on a paint shaker. DNA was extracted from each sample using the CTAB protocol [7].

SSR marker selection, primer design and dye labeling

A total of 74 SSR markers that were previously mapped in soybean [3] were used for the development of multiplex sets. Most of the SSR markers that were included had (ATT) motifs due to their abundance and polymorphic nature in soybean and their easily interpretable allele patterns. The use of SSR markers with mono- or di-nucleotide repeats was minimized due to their associated stutter allele patterns that can complicate accurate genotyping. The original primers for each marker were developed by Cregan *et al.* [3]. New primer sites were chosen to adjust the allele size range for most markers by using cloned genomic DNA sequence information from the soybean cultivar ‘Williams’ that flanked each SSR locus. Previous estimates of the allele size range of 20 SSR markers were used instead of redesigning their primers. Primer sequences were designed with a melting point (T_m) of 60–62 °C and were checked by OLIGO v.5.0 (National Biosciences) to avoid self-complementation or secondary structure. Primers were synthesized on a PE/ABI model 394 or 3948 DNA synthesizer in the

DNA Sequencing and Synthesis Facility at Iowa State University (Ames, IA). Fluorescent amidites, either 6-carboxyfluorescein (6-FAM; blue), hexachloro-6-carboxyfluorescein (Hex; yellow), or tetrachloro-6-carboxyfluorescein (TET; green), were directly attached to the 5′ end of the forward primer during the last cycle of oligonucleotide synthesis. The average primer length was 24-mer.

PCR conditions

All PCR reagents were obtained from Perkin-Elmer Applied Biosystems (PE/ABI). A 10 μl reaction volume contained 1.0 μl of 30 ng genomic DNA, 2.0 mM magnesium chloride, 200 μM of each dNTP, 1.0 unit of AmpliTaq Gold DNA polymerase, and 1.0 μl of GeneAmp 10$\times$ PCR Buffer II. For individual SSR marker analysis, 0.25 ρM each of forward and reverse primer was used. For multiplexing, the primer concentrations varied, as described in the next section (step 3). PCR amplifications were conducted on a GeneAmp model 9600 or 9700 thermocyclers (PE/ABI). The PCR protocol was 95 °C for 10 min followed by 35 cycles of 95 °C for 25 s, 58 °C for 25 s, and 72 °C for 25 s, followed by a final extension at 72 °C for 60 min. The final extension was used to correct for nontemplate addition by *Taq* polymerase of a nucleotide, primarily adenosine, to the 3′ end of amplification products [13].

Multiplex set development and data analysis

Four steps were conducted to develop multiplex sets.

Step 1. The allele size range of each SSR marker was estimated by analyzing pooled DNA samples. Four pooled DNA samples were prepared by combining equal concentrations of DNA from individual genotypes. Two pooled samples were formed with five different PI genotypes, and two were formed with five different elite genotypes. Each pooled DNA sample was amplified with individual markers, as described above. A 1.5 μl volume from each PCR amplification was mixed with 2.4 μl formamide, 0.5 μl blue dextran/EDTA loading dye, and 0.6 μl internal size standard GS-350 (PE/ABI). The size standard contained DNA fragments labeled with the fluorescent amidite N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA; red) and ranged in size from 35 to 300 bp. The sample was heated for 2 min at 95 °C and a 1.3 μl volume was loaded in one lane of a 4.25% polyarcylamide gel mounted on a PE/ABI model 377 automated DNA sequencer. Electrophoresis was carried out at 3000 V for 2 h. Data were collected with DNA Sequencing Collection software version 2.5 (PE/ABI) and analyzed with GENESCAN Prism software version 2.1 (PE/ABI). SSR allele size ranges were estimated with GENOTYPER software version 2.0 (PE/ABI) by scrolling through the electropherogram and manually clicking on the two outermost peaks. Allele sizes were automatically calculated to two decimal places using the local Southern algorithm [5].

Step 2. Multiplex sets were constructed based on the allele size range estimates and the type of forward primer label of markers. Markers that had overlapping allele size ranges, but different labels, were considered for a set. Markers with the same label and allele size ranges separated by more than 20 bp also were considered for a set. Markers with the same label and overlapping allele size ranges were not placed into the same set. Based on these parameters, up to eight SSR markers were included in a set. For multiplex PCR, up to three markers with the same dye label were included in a single amplification.

Step 3. To optimize the multiplex sets for genotyping, four individual DNA samples were analyzed. Initially, equal concentrations of primer were used in multiplex PCR, and equal volumes of PCR product from each reaction were pooled into a single sample. A 1.5 μl volume of the pooled sample was analyzed, as described in Step 1. Primer concentration(s) and the amount of product pooled from PCR amplifications of individual or multiplexed markers were increased or decreased based on the signal intensity of their corresponding allele peak when measured in GENOTYPER.

Step 4. All genotypes were analyzed with each multiplex set to determine if the allele size range among multiplexed markers with the same dye label would overlap. The analysis of individual genotypes also was conducted to develop GENOTYPER templates for semi-automated genotyping.

Results and discussion

Eleven multiplex sets comprising 74 SSR markers that covered the soybean genome were developed (Table 1). The average number of markers per multiplex set was seven. SSR length variants were considered alleles because the markers were previously mapped to a single locus [3]. In addition, the banding, or al-

Table 1. SSR markers included in each multiplex set.

Multiplex set	Number of markers	SSR markers[1]
1	7	GMGLPS12, HSP176, Satt022, Satt063, Satt006, Satt020, Satt009
2	6	Sct_026, Satt155, Satt042, Satt170, Satt165, Satt187,
3	7	Satt072, Satt046, Satt176, Satt173, Satt168, Satt134, Satt160
4	7	Satt184, Satt179, Satt172, Satt190, Satt077, Satt005, Satt141
5	7	Satt070, Satt185, Sctt008, Satt135, Satt012, Satt045, Satt014
6	8	Satt161, Satt171, Satt178, Satt148, Satt183, Sctt009, Satt181, Satt001
7	6	Satt189, Satt152, Satt146, Satt143, Satt182, Satt175,
8	6	Satt186, Satt154, Satt129, Satt153, Satt031, Satt002,
9	7	Satt225, Satt588, Satt271, Satt367, Satt309, Satt307, Satt285
10	7	Satt384, Satt336, Satt194, Satt242, Satt329, Satt409, Satt314
11	6	Satt358, Satt395, Satt227, Satt390, Satt577, Satt357

[1]SSR markers with an 'S' prefix were obtained from the USDA/ARS Beltsville Agricultural Research Center. The suffix of these markers indicates the repeat type (e.g. att). Other SSR markers were retrieved from GenBank.

lele peak pattern of each marker was in accordance with the amplification of a single locus from a highly homogeneous species where greater than 90% of the genotypes possessed a single allele at each marker locus. For those infrequent cases where an individual genotype had more than one allele, it was assumed that the genotype was derived from a plant that was heterozygous for that marker locus.

At least one of the 74 SSR markers occurred on each of the 20 linkage groups of soybean reported by Cregan *et al.* [3] with an average of four markers per linkage group. The location of each marker on the soybean linkage map is shown in Figure 1. Some of the linkage groups contained several markers that were relatively evenly spaced, based on estimated genetic distance (e.g. A2, C2, K), whereas other did not (e.g. B1, J, K).

The analysis of a single genotype with the 74 SSR markers required only 11 gel lanes and 40 PCR runs reducing the cost of PCR reagents by ca. 45% and gel costs by ca. 85% compared with conventional procedures. Greater use of multiplex PCR by incorporating more markers into a single PCR amplification could further reduce this cost. In this study, the most time consuming step was the optimization of multiplex PCR. The current version of the primer design software program OLIGO (v. 6.0) has features to facilitate the development of multiplex PCR protocols. Extensive use of multiplex PCR could, however, limit the flexibility of a multiplex set whereby additions or substitutions of markers may require extensive reoptimization.

Grouping SSR markers into multiplex sets depends on the dye label of the markers and their allele size range. The first step in multiplex development, primer labeling, is critical because it chiefly determines the number of markers that can be grouped together in a multiplex set. It is also a costly step because the dye labels are relatively expensive. In this study, the primer sites for most of the SSR markers were placed physically closer to or further away from the core repeat site to alter the allele size range. This separated the allele size ranges of several marker loci with similar SSR repeat motifs, which increased the number of markers that could be included in the same multiplex set.

The analysis of pooled DNA samples from both PI and elite soybean genotypes was conducted to provide robust estimates of the allele size range of marker loci. The allele size range between these two groups of germplasm differed by ca. 20 bp or more for ca. 30% of the markers. For example, Figure 2 shows the analysis of pooled DNA samples for marker Satt367. The allele size range detected in the PI samples (Panels A and B) was from $\approx$ 193 to 230 bp whereas the range detected in the elite samples was only from $\approx$ 211 to 221 bp (Panels C and D).

The initial screening of four individuals with each multiplex set showed extensive variability in the signal intensity of allele peaks from markers within a set. Several markers yielded allele peaks with signal intensities $\approx$6000 fluorescent units, whereas others were not detectable in GENOTYPER. This was primarily corrected by adjusting the primer concentration in multiplex PCR and/or by increasing the amount of PCR product pooled so as not to reduce the amount of

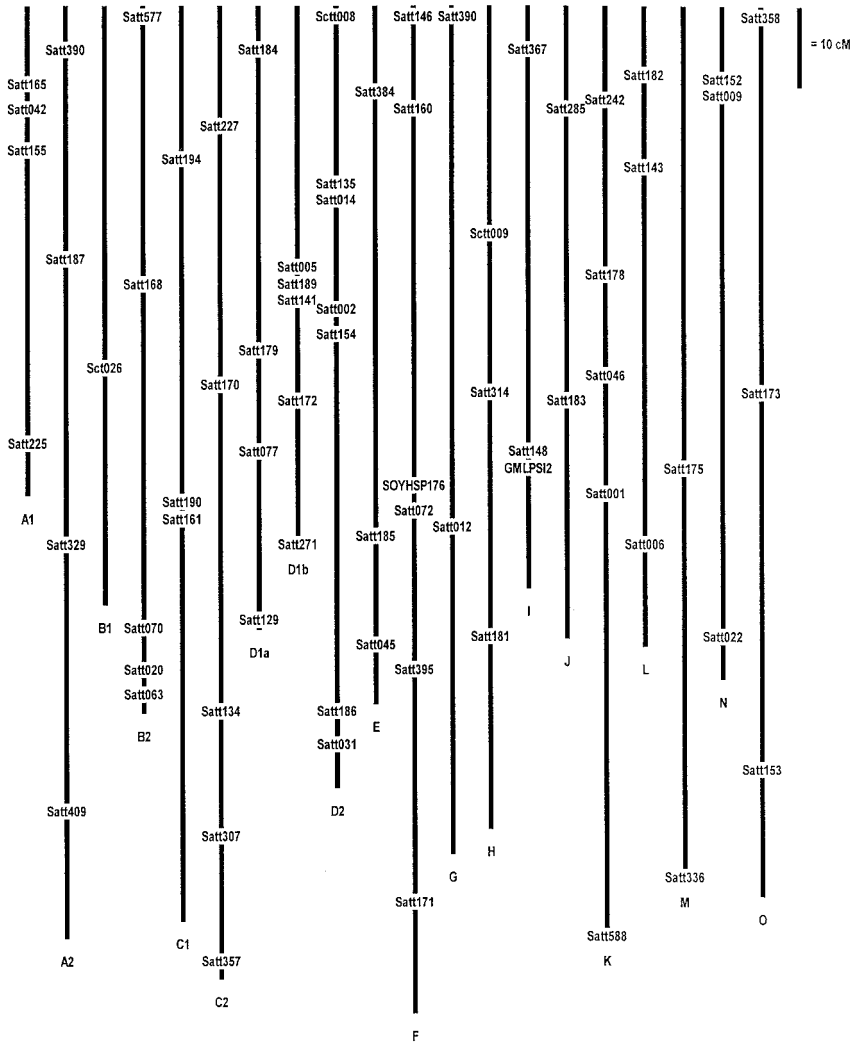

Figure 1. Approximate distribution of the 74 SSR markers on the soybean linkage map [3]. The bottom of the linkage groups are labeled with their corresponding letter designations. The linkage groups are drawn to scale, in terms of genetic distance, with an estimated length of 10 cM indicated in the upper right-hand corner.

multiplex PCR. In some cases, markers had to be amplified individually to yield interpretable allele peak patterns. These optimization steps greatly improved the accuracy of allele scoring in GENOTYPER. An example of an electropherogram for multiplex set six is shown in Figure 3 with the allele size range for each SSR marker and multiplexing conditions described in Table 2. This figure also demonstrates how alleles with the same size, but from different markers, are analyzed. As shown in the figure, two 93 bp alleles, one from Satt178 (TET-labeled; green) and the other from Satt181 (HEX-labeled; yellow) were detected. Because different dye labels were used, each allele was scored for its corresponding marker.

The 79 genotypes were analyzed with each multiplex set to ensure that the allele size range among multiplexed SSR markers with the same label would not overlap. Additional alleles were detected, but only two markers within each of two sets overlapped. In one case, this was corrected by substituting one marker into a different set. In the second case, a substitution could not be easily made. To correct for this overlap, the reverse primer for one of the markers was placed further away from the SSR to eliminate the overlap between the two markers. The smallest difference in allele size range among multiplexed markers with the same forward primer label was 9 bp, and the average minimum difference was 42 bp. The threshold for the

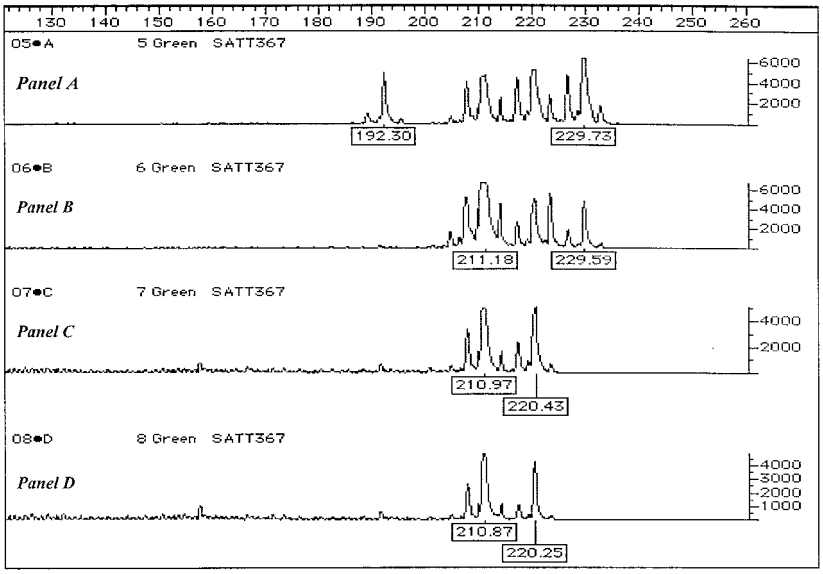

Figure 2. Electropherogram display of the analysis of pooled DNA samples for marker Satt367 taken from GENOTYPER output. A. Analysis of a pooled sample of five PI genotypes. B. A pooled sample of five different PI genotypes. C. Analysis of a pooled sample of five elite genotypes. D. A pooled sample of five different elite genotypes. The top-horizontal scale is a size range in bp and the right-vertical scale is a measure of intensity, in fluorescent units, of the allele peaks. The allele size range for Satt367 was estimated from the two outermost peaks across all samples (192 to 230 bp).

Table 2. Description of SSR marker loci in multiplex set six including their dye label, linkage group designation, allele size range, grouping for PCR, and the amount of product pooled from each PCR amplification.

Dye label[1]	Marker locus	Linkage group[2]	Allele size range (bp)[3]	PCR amplification[4]	PCR pool (μl)[5]
FAM	Satt161	C1	101–156	1	
					1
FAM	Satt171	F	247–277	1	
TET	Satt178	K	93–126	2	1
TET	Satt148	I	150–171	3	
					2
TET	Satt183	J	219–243	3	
HEX	Sctt009	H	86–93	4	1
HEX	Satt001	K	103–145	5	
					3
HEX	Satt181	H	167–228	5	

[1]The 5′ label of the forward primer of each marker.
[2]Linkage group designations correspond to the ISU/USDA genetic map [3].
[3]Allele size range determined from the analysis of 79 soybean genotypes.
[4]Markers followed by the same number are amplified in the same PCR amplification.
[5]The volume of PCR product indicated for each PCR amplification is pooled into a single sample. A 1.5 μl volume of the pooled sample is used for analysis.

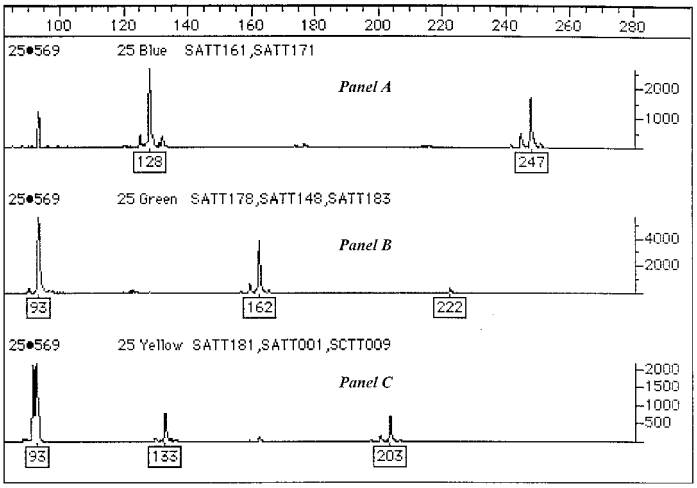

Figure 3. Electropherogram display of the analysis of eight SSR markers from multiplex set six in a single gel lane taken from GENOTYPER output. SSR markers with the same label are analyzed in individuals panels. A. Analysis of two FAM (blue) labeled SSR markers. B. Three TET (green) labeled SSR markers. C. Three HEX (yellow) labeled SSR markers. The top-horizontal scale is a size range in bp and the right-vertical scale is a measure of intensity, in fluorescent units, of the allele peaks. Each peak is labeled with the size of the allele (bp).

allowable minimum difference in allele size range between markers in the same set that have the same label should be based on the extent evaluation. In this study, two different groups of soybean germplasm representing 79 diverse genotypes were evaluated. Because of this extensive evaluation, the two markers with the same label and allele size range separated by only 9 bp were kept in the same multiplex set. If fewer or less diverse genotypes are used for multiplex development, the minimum distance should be increased.

The analysis of the 79 genotypes enabled the development of GENOTYPER templates, a database of collected allele size information for each marker locus within a multiplex set that can be used for semi-automated genotyping. An example of a template for multiplex set six is shown in Figure 4. Each SSR marker locus was classified as a group, and the alleles at that locus are categories of the group. The categories (alleles) were defined by their color and the breadth of their peak. When a new allele was identified, the peak range at which it occurred was measured at its base, recorded, and used to categorize the allele in whole bp numbers. For semi-automated analysis, the templates are run as a macro in GENOTYPER, and, if an individual produces a peak for a particular marker that has the color and size range of a category, the peak is scored as an allele. This can be interpreted by evaluating Figures 3 and 4. Figure 3 (panel C) shows three yellow-labeled peaks. The peak that was scored as a 203 bp allele fits this category from Satt001 as indicated in Figure 4. This feature would be advantageous for analyzing mapping populations derived from parents whose allele data are stored in GENOTYPER templates. This method of analysis is considered semi-automated because manual inspection of peaks on an electropherogram is needed to ensure accurate allele size scoring by the software. The information generated by GENOTYPER templates can be incorporated into tables and stored in EXCEL format.

Multiplex SSR marker technology facilitates high-throughput, accurate, and cost-effective genotyping. The use of the GENOTYPER templates developed in this study should expedite future analyses while maintaining a level of accuracy that would far exceed that from conventional analysis. A description of all multiplex sets including PCR and pooling conditions, primer sequences, and examples of electropherogram displays are provided in Soybase, the USDA-ARS-sponsored genome database (http//129.186.26.94/publication_data/Narvel/multiplex.html). The primer sequences of SSR markers in multiplex sets 9–11 are the same as those previously reported [3].

Because the multiplex sets were constructed by using diverse germplasm, they should be applicable for genotyping most soybean populations without the occurrence of allele overlap. This would make the multiplex sets particularly useful for conducting diversity analysis, determining pedigree relationships, genotyping for proprietary purposes, or in a backcross-

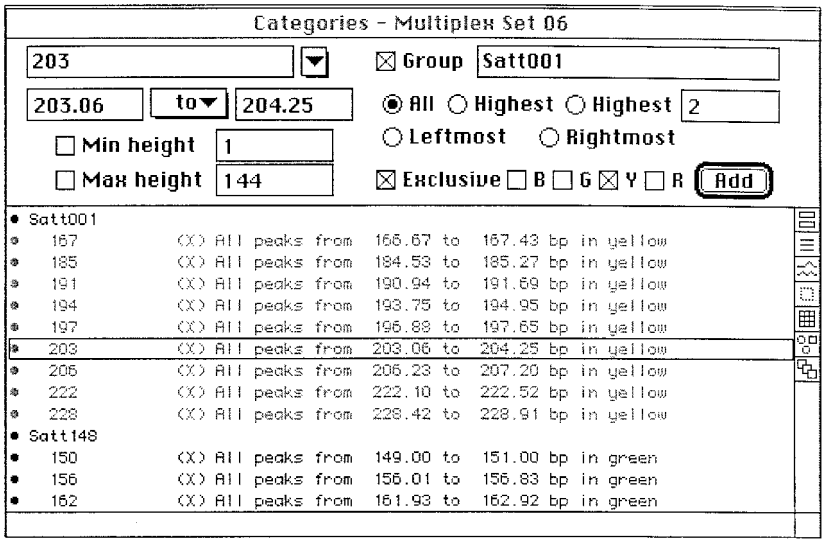

Figure 4. An example of a GENOTYPER template for multiplex set six. The top of the figure describes the parameters used to define each category (allele). The bottom of the figure is a list of categories under SSR marker Satt001 and a partial list under Satt148.

introgression scheme to selectively recover the recurrent parent. New alleles that may be detected in future studies could easily be added to the current GENOTYPER templates. Additional SSR markers could be added to the existing multiplex sets to increase genome coverage that would make the multiplex sets useful for linkage mapping. Since development of these multiplex sets, several hundred more SSRs have been identified in soybean [3]. Some of the methods used in this study could be conducted with these SSRs to enhance our genotyping system.

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